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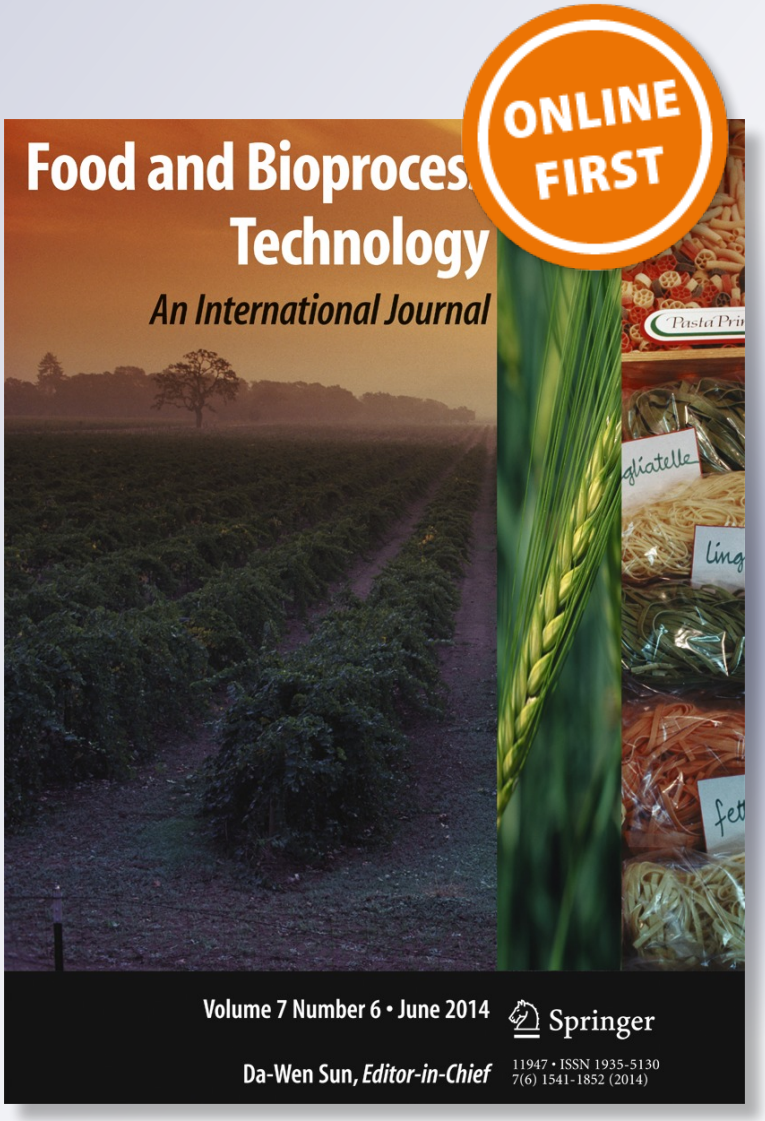

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ORIGINAL PAPER

Antimicrobial Polylactic Acid Packaging Films against Listeria and Salmonella in Culture Medium and on Ready-to-Eat Meat

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Abstract The contamination of *Listeria monocytogenes* and *Salmonella* spp. in ready-to-eat (RTE) meat products has been a concern for the meat industry. In this study, edible chitosan-acid solutions incorporating lauric arginate ester (LAE), sodium lactate (NaL), and sorbic acid (SA) alone or in combinations were developed and coated on polylactic acid (PLA) packaging films. Antimicrobial effects of coated PLA films on the growth of *Listeria innocua*, *L. monocytogenes*, and *Salmonella* Typhimurium in a culture medium (tryptic soy broth, TSB) and on the surface of meat samples were investigated. Antimicrobial PLA films containing 1.94 mg/cm^2 of chitosan and 1.94 μg/cm^2 of LAE were the most effective against both *Listeria* and *Salmonella* in TSB and reduced them to undetectable level (<0.69 log CFU/ml). The same PLA films with LAE significantly ($p<0.05$) reduced the growth of *L. innocua*, *L. monocytogenes*, and *S.* Typhimurium on RTE meat during 3 and 5 weeks' storage at 10 °C, achieving 2–3 log reduction of *Listeria* and 1–1.5 log reduction of *Salmonella* as compared with controls. PLA films coated with 1.94 mg/cm^2 of chitosan, 0.78 mg/cm^2 of NaL, and 0.12 mg/cm^2 of SA significantly reduced the growth of *L. innocua* but were less effective against *Salmonella*. The combination of NaL (0.78 mg/cm^2) and SA (0.12 mg/cm^2) with LAE (1.94 μg/cm^2) did not generate additional or synergetic antimicrobial effect against *Listeria* or *Salmonella* on the meat surface. *L. innocua* had a similar sensitivity to the film treatments as *L. monocytogenes*, suggesting that *L. innocua* may be used as a surrogate of *L. monocytogenes* for further scaleup and validation studies. The film treatments were more effective against the microorganisms in TSB culture medium than in RTE meat, which suggests that in vivo studies are a necessary step to develop antimicrobial packaging for applications in foods.

Keywords Antimicrobial packaging · Polylactic acid film · Lauric arginate · *Listeria* · *Salmonella* · RTE meat

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Introduction

Since the nineteenth century, food packaging technology has made great advances as a result of global trends and consumer preferences. These advances are oriented to obtain improved food quality and safety. Moreover, with the movement toward globalization, food packaging also requires longer shelf life, along with the monitoring of safety and quality based upon international standards. Antimicrobial food packaging is a packaging system that is capable of killing pathogenic microorganisms that contaminate the foods or inhibiting spoilage caused by mishandling or faulty packaging. The antimicrobial function can be achieved by adding antimicrobial agents into the packaging system and/or using antimicrobial polymers that satisfy conventional packaging requirements.

Biopolymers are drawing attention from many researchers especially because of their petroleum-independent sources and eco-friendliness. Polylactic acid (PLA) is biosourced and biodegradable; therefore, the use of PLA in antimicrobial

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packaging could provide additional environmental benefits as compared to petroleum polymers. Since the first large-scale PLA production facility became a reality in 2002, PLA has gradually gained market importance. PLA has been used in a wide range of applications, such as food packaging materials (cups, films, and trays), textiles (shirts), nonwoven fabrics (diapers) (Jamshidian et al. 2010), as well as antimicrobial packaging materials (Jin and Niemira 2011; Jin and Zhang 2008; Liu et al. 2010, 2009).

Chitosan, a deacetylated derivative of chitin, is the second most abundant polysaccharide found in nature after cellulose and has been proved to be nontoxic, biodegradable, biofunctional, and biocompatible (Kong et al. 2010). As a packaging material, chitosan brings some advantages over other biomolecule-based active polymers because of its antimicrobial activity, bivalent mineral-chelating ability, and film-forming capacity; however, poor mechanical and gas barrier properties and weak water resistance limit its application particularly in the presence of water and humidity (Wang et al. 2005; Xu et al. 2005). Therefore, chitosan could not be used as a "stand alone" packaging material.

The recognition of the potential toxicity of synthetic antimicrobials and the health benefits attributed to many natural compounds has encouraged the quest for natural antimicrobial agents. Lauric arginate ester (LAE) is a generally recognized as safe (GRAS) food additive by the US Food and Drug Administration and is metabolized rapidly to naturally occurring amino acids, mainly arginine and ornithine, after consumption (Ruckman et al. 2004). Sodium lactate (NaL) is primarily used as a flavor enhancer in meat and poultry products (Shelef 1994). Organic acids have long been used as food additives or preservatives. The antimicrobial activity of sorbic acid as well as its salt against *Listeria monocytogenes* has been studied in laboratory media and in foods such as cheese, meat products, or fish (Dorsa et al. 1993; Samelis et al. 2001).

Antimicrobials (AM) in polymers can be coated on the surface of food (direct coating) or the surface of packaging materials (indirect coating). Antimicrobial films or coatings have been found to be more effective than the addition of antimicrobial agents directly to food as these may gradually migrate from the package onto the surface of the food, providing concentrated protection when most needed (Durango et al. 2006; Jin and Zhang 2008; Jin et al. 2009b; Zhang et al. 2004). In our previous studies, chitosan-acid solutions incorporating antimicrobials were used for direct coating of food surfaces (Chen et al. 2012; Guo et al. 2013a, b; Jin and Gurtler 2012; Jin et al. 2013). Although the direct coating method is very effective to reduce pathogens and spoilage microorganisms on food surfaces, additional packages are needed for final products. Antimicrobial-coated PLA films provide one-step packaging for the food industry. The use of chitosan for coating different films has been reported. Miltz et al. (2006) studied the effectiveness of a corn starch-based film coated with peptide dermaseptin S4 derivative as an AM agent against molds and aerobic bacteria on cucumbers. Coma et al. (2001) found that cellulose films coated with nisin inhibited *Listeria innocua* and *Staphylococcus aureus* on laboratory media. Chen et al. (1996) prepared AM films containing 2 or 4 % (*w*/*w*) of sodium benzoate and potassium sorbate by casting methylcellulose, chitosan, and their mixtures. Ming et al. (1997) reported that a cellulose casing coated with pediocin completely inhibited the growth of *L. monocytogenes* on ham, turkey breast, and beef products for 12 weeks at 4 °C. Janes et al. (2002) investigated the antimicrobial effect of corn zein films coated with nisin and/or 1 % (*w*/*w*) calcium propionate against *L. monocytogenes* inoculated on ready-to-eat chicken samples and found that the coated films inhibited the growth of the microorganism. Kim et al. (2008) evaluated the effectiveness of chitosan and whey protein insolate coated with lysozyme against the growth of *L. monocytogenes* and *Salmonella enteritidis* inoculated on hard-boiled eggs. Siragusa and Dickson (1992, 1993)) found that calcium alginate coatings and films containing organic acids effectively reduced the populations of *L. monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 on the surface of beef carcass.

However, little has been reported on the activity of antimicrobials coated on biodegradable polymers; in addition, most of them used a single acid, such as acetic acid, for making chitosan solutions and few reported the combination of multiple organic acids, LAE, NaL, sorbic acid (SA), and PLA film. Therefore, the objective of this study was to develop effective formulas for coating on PLA films and investigate the antimicrobial efficacy in a microbial culture medium and ready-to-eat (RTE) meat. *Listeria* (Gram positive) and *Salmonella* (Gram negative) were used as representatives of food-borne pathogens.

Materials and Methods

Materials

Chitosan (low molecular weight, 150 kDa, 75–85 % deacetylation) and sorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Food grade acetic acid, citric acid, lactic acid, and levulinic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). LAE solution (CytoGuard®) containing 20 % LAE was from A&B Ingredients (Fairfield, NJ, USA). Sodium lactate (sodium DL-lactate) was purchased from Spectrum Chemical MFG (Gardena, CA, USA). PLA films (EVLON Heatseal) were from BI-AX International Inc. (Ontario, Canada). The PLA films have

0.2 mm thickness, density of ca. 1.24 g/cm^3, and seal initiation temperate of ca. 80 °C.

Preparation of the Chitosan Coating Solution and Film Coating

The coating solutions were prepared by mixing 2 or 5 % chitosan in acid solutions containing 2 % of acetic acid (AA), citric acid (CA), lactic acid (LA), and levulinic acid (LevA), or their combinations. LAE, NAL, and SA, single or in combination at predetermined concentrations as shown in Table 1, were added to a chitosan-acid mixture. Each mixture was stirred with a magnetic stir bar on a stir plate until the polymer was completely dissolved.

PLA films (10×18 cm^2) were coated with 7 ml of each coating solution, and the coated PLA films were then vacuum-dried at 50 °C for 2 h. The concentrations of chitosan, LAE, sodium lactate, and sorbic acid per square centimeter on each coated film are listed in Table 1.

Culture Preparation

L. innocua (ATCC 51742, 33090, and 33091), *L. monocytogenes* (HAT2, Scott A, JBL2365), and *S.* Typhimurium (Beef Isolate, Enteritidis, St. Paul) were obtained from the culture collection of the US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center (Wyndmoor, PA, USA). Frozen stock cultures of each strain were cultured independently in 30 ml tryptic soy broth (TSB, BBL/Difco Laboratories, Sparks, MD, USA) in sterile 50-ml conical tubes at 37 °C for 18 h. Equal-volume aliquots from each culture were then combined to make a cocktail.

Antimicrobial Effect of Films on the Growth of Listeria and Salmonella in TSB

Chitosan-coated PLA films (1×2.6 cm^2) were placed into culture tubes (16×150 mm, Fisher Scientific) containing 10 ml of TSB, and the tubes were immediately inoculated with the *L. innocua* or *S.* Typhimurium culture prepared above to a final concentration of 10^{3-4} colony forming units (CFU)/ml. The TSB tubes were then incubated at 22 °C for 0, 24, and 48 h with shaking at 100 rpm. To determine the populations in TSB after incubation, serial dilutions of the resultant bacterial suspensions were made in 0.1 % peptone water and surface-plated (100 μm) onto PALCAM agar plates (BBL/Difco) with PASLCAM selective supplement (Oxoid, England) for *Listeria* and XLT4 agar plates (BBL/Difco) with XLT4 selective supplement (BBL/Difco) for *Salmonella*, and then incubated at 37 °C up to 48 h. The CFU were then enumerated.

Antimicrobial Effect of the Chitosan-Coated Film on the Growth of Listeria and Salmonella on RTE Turkey Meat

Meat Sample Preparation

Presliced turkey deli meat labeled as no preservatives was purchased from a local grocery store; the thickness was approximately 1 cm. Meat slices were cut into 4×4 cm squares, vacuum-packaged, and stored in a freezer (−20 °C). Prior to experiments, the meat samples were thawed overnight in a refrigerator (4 °C).

Film Treatment

Meat samples were placed on a sterile tray in a biological hood, and the upper surface (3×3 cm) of each sample was inoculated with 0.1 ml of *L. innocua*, *L. monocytogenes*, or *Salmonella* cocktails. The inoculum was then spread evenly over the surface (3×3 cm) using sterile spreaders (Fisher Scientific, Fair Lawn, NJ, USA). After inoculation, samples were kept under the biohood for 2 h to allow bacterial attachment. For PLA film treatment, one piece of coated PLA film (approximately 4×4 cm each) was put on the top of each inoculated RTE turkey slice, and then samples were packed into a vacuum pouch (152.4×203.2×0.08 mm; polynylon; Uline, Inc., Waukegan, IL, USA) and vacuum-sealed in a vacuum sealer (Model V-300, Fuji Impulse Co., Japan). Inoculated samples without antimicrobial PLA film and inoculated samples with non-coated PLA film also were vacuum-packaged and served as controls. All meat samples were stored at 10 °C to simulate mild temperature abuse.

Microbiological Analysis

Untreated and treated meat samples were transferred into individual sterile stomacher bags and then hand-massaged in 20 ml of 0.1 % peptone water for 1 min. Sterile dilutions of the resultant bacterial suspensions were made in 0.1 % peptone water and surface-plated (100 μl) onto PALCAM or XLT4 plates and then incubated at 37 °C up to 48 h. The CFU were then enumerated.

Statistical Analysis

All experiments were replicated independently at least three times, with duplicate samples prepared for each experimental trial. The CFU per milliliter or CFU per square centimeter numbers were transformed to $\log_{10}$ values and analyzed using analysis of variance with SAS version 9.1 software (SAS Institute, Carry, NC, USA). Duncan's multiple range test was used to determine the significant differences between treatment means. The significance level was set at α=0.05.

Table 1 Formulation of coating solutions for packaging films

Formula	Codes of film	Chitosan (mg/cm^2)	Lauric arginate ester ($\mu g/cm^2$)	Sodium lactate (mg/cm^2)	Sorbic acid (mg/cm^2)
Control	CK	–	–	–	–
2 % chitosan	2CHI	0.39	–	–	–
5 % chitosan	5CHI	1.94	–	–	–
5 % chitosan+5 % LAE	5CHI5LAE	1.94	1.94	–	–
5 % chitosan+10 % LAE	5CHI10LAE	1.94	3.89	–	–
5 % chitosan+10 % NaL	5CHI10NaL	1.94	–	3.89	–
5 % chitosan+20 % NaL	5CHI20NaL	1.94	–	7.78	–
5 % chitosan+2 % NaL+0.3 % SA	5CHI2NaL03SA	1.94	–	0.78	0.12
5 % chitosan+4 % NaL+0.6 % SA	5CHI4NaL06SA	1.94	–	1.56	0.23
5 % chitosan+5 % LAE+2 % NaL+0.3 % SA	5CHI5LAE2NaL0.3SA	1.94	1.94	0.78	0.12

Results

Antimicrobial Efficacy of PLA Films Containing Two Concentrations of Chitosan in Acid Solution on the Growth of L. innocua in TSB

As reported by Gurtler et al. (2012), two or three acids in combination, particularly the combination of LA, AA, and LevA, exhibited significant antimicrobial efficacy against *Salmonella*. Our previous studies also demonstrated that coating solutions containing these three-acid combinations and 2 % chitosan had very effective antimicrobial activities against *Listeria* and *Salmonella* in various foods (Chen et al. 2012; Guo et al. 2013a, b; Jin and Gurtler 2012). Therefore, two or three acids in combination were investigated in our first trials, using this three-acid combination as a baseline. Figure 1a shows PLA films coated with 2 % chitosan in five acid combinations against the growth of *L. innocua* in TSB at 22 °C for 48 h. While *Listeria* in control samples grew to 7.2 log after 24 h and to 9.2 log after 48 h, all the film treatments significantly ($p<0.05$) inhibited the growth of *Listeria* after 24 h. However, the film with CA+LevA showed the least inhibitive effect after 48 h. Therefore, this treatment was excluded from the next trial. Figure 1b shows the results when 5 % chitosan was used with the rest of the four acid combinations against *Listeria*. The increase of chitosan concentration from 2 to 5 % significantly ($p<0.05$) increased the antimicrobial activity of all treatments. Both LA+LevA and LA+LevA +AA reduced 1.5 log of *Listeria* while both CA+LA and LA +CA+LevA inhibited the growth of *Listeria* within 48 h. In two-acid combinations, LA+LevA had higher antimicrobial activity than LA+CA, and in three-acid combinations, LA+ LevA+AA was more effective than LA+LevA+CA. Therefore, LA+LevA and LA+LevA+AA with 5 % chitosan were used for further experiments.

Antimicrobial Efficacy of PLA Films Containing Chitosan and LAE Against L. innocua and S. Typhimurium in TSB

LAE at two concentrations (1 and 2 %) was added in the coating solutions containing 5 % chitosan with LA+LevA or LA+LevA+AA. The coated films had an equivalence of 1.94 mg/cm^2 of chitosan and 1.94 $\mu g/cm^2$ of LAE. Figure 2 shows the survivals of *L. innocua* and *S.* Typhimurium in TSB after these film treatments.

Both *Listeria* and *Salmonella* in controls grew to over 9 log CFU/ml after 48 h. All four film treatments reduced both bacteria to undetectable levels (<0.69 log CFU/ml) after the treatments and inhibited the growth for 48 h, suggesting that LAE in the films is effective against both *Listeria* (Fig. 2a) and *Salmonella* (Fig. 2b) in TSB.

Antimicrobial Efficacy of PLA Films Containing Chitosan and NaL Against L. innocua and S. Typhimurium in TSB

NaL at two concentrations (10 and 20 %) was added to the coating solutions containing 5 % chitosan with LA+LevA or LA+LevA+AA. The coated films had an equivalence of 1.94 mg/cm^2 of chitosan and 3.89 or 7.78 mg/cm^2 of NaL. The survivals of *Listeria* and *Salmonella* in TSB after those film treatments are shown in Fig. 3.

All the treatments completely inhibited the growth of *Listeria* for 24 h, whereas control samples had 7 log CFU/ml of *Listeria*. The population of *Listeria* increased ca. 0.5 log from 24 to 48 h in all film-treated samples. Higher NaL concentration in the films had less growth of *Listeria* at 48 h although there were no statistical differences among them (Fig. 3a).

Similar results were obtained for *Salmonella* at 24 h (Fig. 3b). However, *Salmonella* in all film-treated samples grew to 6~7 logs at 48 h, indicating that NaL in the coating solutions was less effective against *Salmonella* than *Listeria*. There were no significant differences among these treatments at 24 and 48 h.

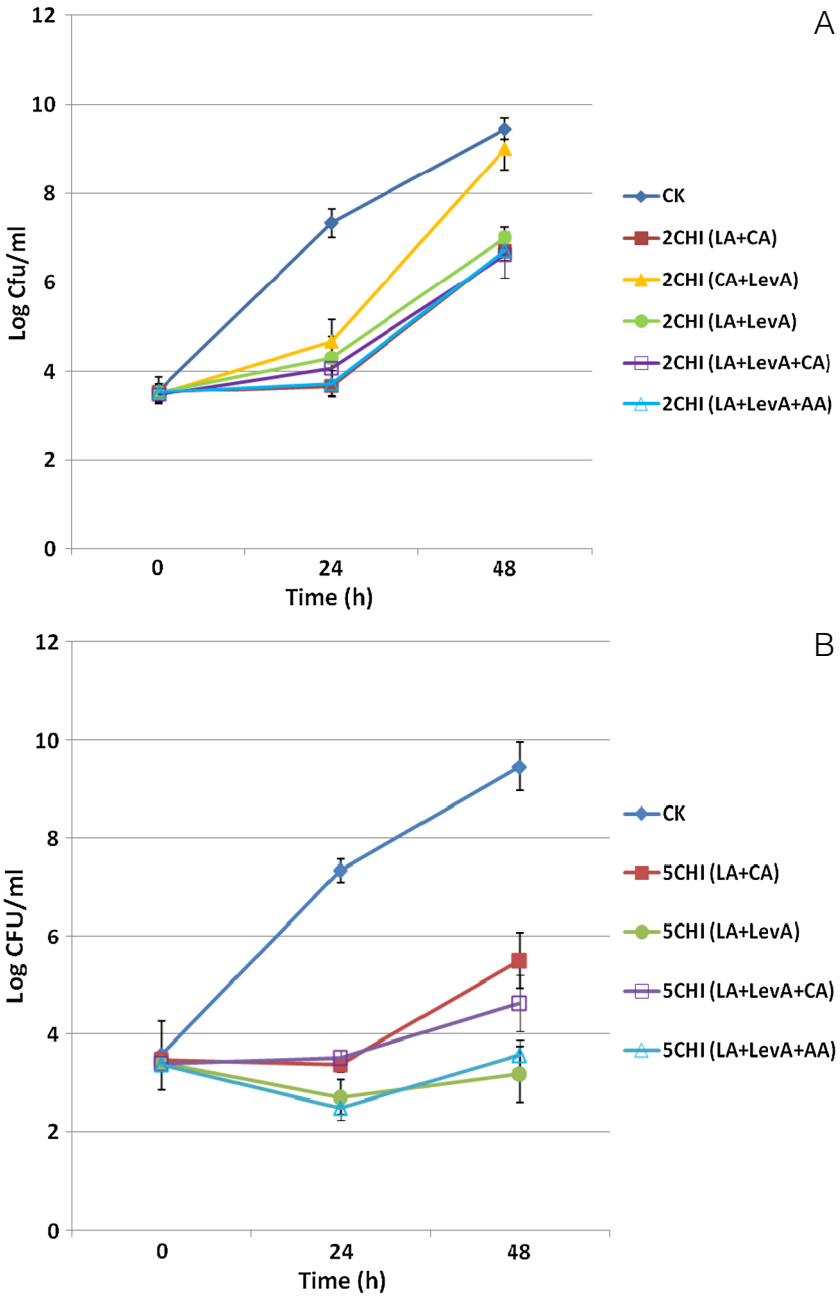

Fig. 1 Growth of *L. innocua* in TSB at 22 °C after treatments of PLA films coated with 2 % chitosan in five acid combinations (**a**) and 5 % chitosan in four acid combinations (**b**). *LA* lactic acid, *LevA* levulinic acid, *CA* citric acid, *AA* acetic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

Antimicrobial Efficacy of PLA Films Containing Chitosan, LAE, NaL, and SA Against L. innocua and S. Typhimurium in TSB

Antimicrobial efficacy of films containing chitosan, LAE, NaL, and SA were further studied. The concentration of LAE at 5 % was used, and NaL concentrations were reduced to 4 and 2 % in combination with 0.3 or 0.6 % SA. The purpose of this experiment was to find any synergetic effect when those antimicrobials were used at lower concentrations in the coating solutions. The films had an equivalence of 1.94 mg/cm^2 of chitosan, 3.89 μg/cm^2 of LAE, 0.78 mg/cm^2 of NaL, and 0.12 mg/cm^2 of SA.

As shown in Fig. 4a, two films with LAE reduced *Listeria* to undetectable levels from 0 to 48 h. Other four film treatments significantly inhibited the growth of *Listeria* at 24 and 48 h. Among them, the treatment with higher NaL and SA concentrations exhibited slightly more effectiveness in inhibitions. Compared with 10 or 20 % NaL used (Fig. 3a), the combination of 4 % NaL+0.6 % SA achieved similar antimicrobial effectiveness against the growth of *Listeria*.

The film treatments with LAE (Fig. 4b) reduced *Salmonella* to undetectable levels at 0, 24, and 48 h; other treatments significantly reduced the growth of *Salmonella* at 24 h, as compared with controls. Two treatments with higher concentrations of NaL and SA also had less growth of

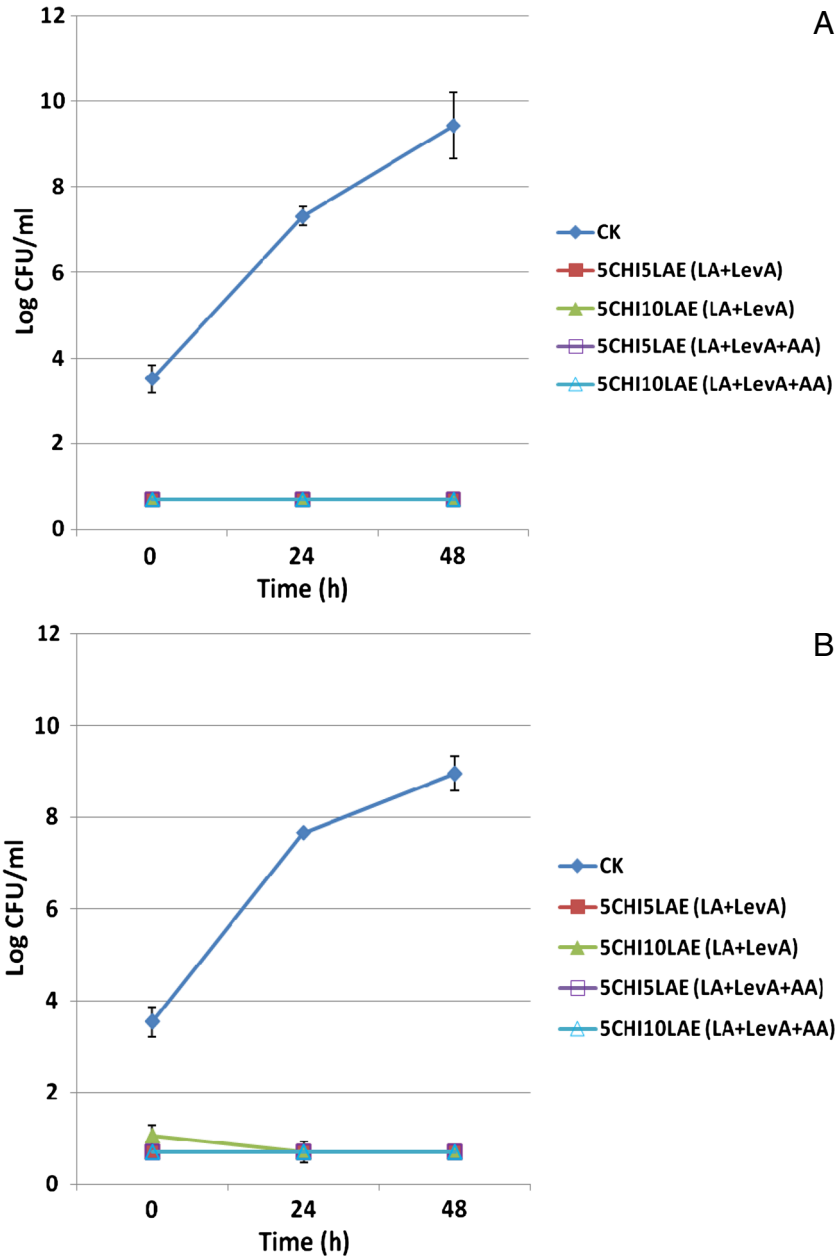

Fig. 2 Survivals of *L. innocua* (**a**) and *S.* Typhimurium (**b**) in TSB at 22 °C after film treatments containing chitosan and LAE. *LA* lactic acid, *LevA* levulinic acid, *AA* acetic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

Salmonella (Fig. 4b). However, the combination of NaL+SA did not exhibit the same or better antimicrobial effectiveness against the growth of *Salmonella* as compared with 10 % NaL (Fig. 3b). Furthermore, the addition of AA in acid solutions did not show significantly additional or synergetic antimicrobial effect.

Antimicrobial Efficacy of PLA Films Containing Chitosan, LAE, NaL, and SA on the Growth of L. innocua on the Surface of Ready-to-Eat Meat

Based on these experiments, two antimicrobial films that were most effective against both *Listeria* and *Salmonella* were selected to validate their antimicrobial efficacy in RTE meats. The PLA film coated with 5CHI5LAE2NaL0.3SA (LA+LevA) and the PLA film coated with 5CHI5LAE (LA+LevA) were used to treat the surface-inoculated meat samples.

As shown in Fig. 5, the initial populations of *L. innocua* inoculated on the meat surface were approximately 5.5 log CFU/cm^2. The populations of *L. innocua* in controls slightly increased to 6.2 log CFU/cm^2 and 6.5 log CFU/cm^2 stored at 10 °C for 24 and 48 h, respectively, while two film treatments significantly reduced the growth of *L. innocua* on the meat surface by 3 log CFU/cm^2 at 24 and 48 h. There was no significant difference between the two films in the antimicrobial activity against *L. innocua* on meat.

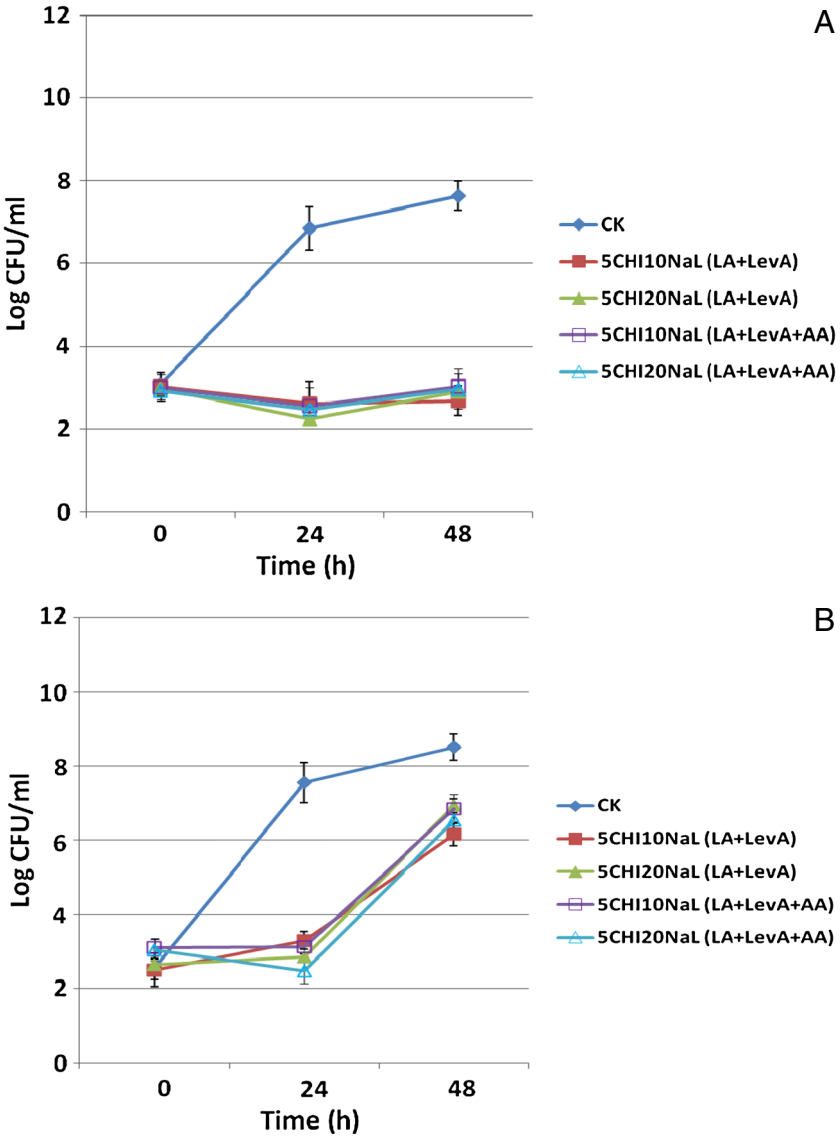

Fig. 3 Survivals of *L. innocua* (**a**) and *S.* Typhimurium (**b**) in TSB at 22 °C after film treatments containing chitosan and NaL. *LA* lactic acid, *LevA* levulinic acid, *AA* acetic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

Antimicrobial Efficacy of PLA Films Containing Chitosan, LAE, NaL, and SA on the Growth of L. monocytogenes on the Surface of Ready-to-Eat Meat

Two additional experiments were conducted to verify the antimicrobial activity of the two films against pathogenic *L. monocytogenes* on the meat surface. One experiment was carried out at a higher inoculation level (ca. 6 log CFU/cm^2) for 3 weeks, and the other was done at a lower level (ca. 4 log CFU/cm^2) for 5 weeks (Fig. 6) at 10 °C.

At both inoculation levels, the two films reduced 2.5–3 log CFU/cm^2 of *L. monocytogenes* and significantly inhibited the growth of *L. monocytogenes* during storage of 3 or 5 weeks at 10 °C. Film-treated meat samples with higher initial populations of *L. monocytogenes* had similar survivals of *Listeria* cells (4 log CFU/cm^2) to those with lower populations after 3 weeks, indicating the initial populations did not significantly affect the effectiveness of the films against *L. monocytogenes*. The survivals of *L. monocytogenes* in film-treated meat samples remained less than 4 log during the extension of 2 weeks' storage while control samples reached over 6 log CFU/cm^2 (Fig. 6b), which suggested that the films could effectively inhibit the growth of *L. monocytogenes* on meat for at least 5 weeks at 10 °C. Similar to *L. innocua*, there was no significant difference between the two films in the antimicrobial activity against *L. monocytogenes* on meat, indicating that the addition of NaL and SA at the tested concentrations did not make extra contribution to reduce or inhibit the growth of *Listeria* on RTE meat.

Antimicrobial Efficacy of PLA Films Containing Chitosan, LAE, NaL, and SA on the Growth of S. Typhimurium on the Surface of Ready-to-Eat Meat

Similar to *L. monocytogenes*, *S.* Typhimurium was inoculated on meat surface at two initial population levels (5 and 3.5 log CFU/cm^2) and stored at 10 °C for 3 and 5 weeks, respectively.

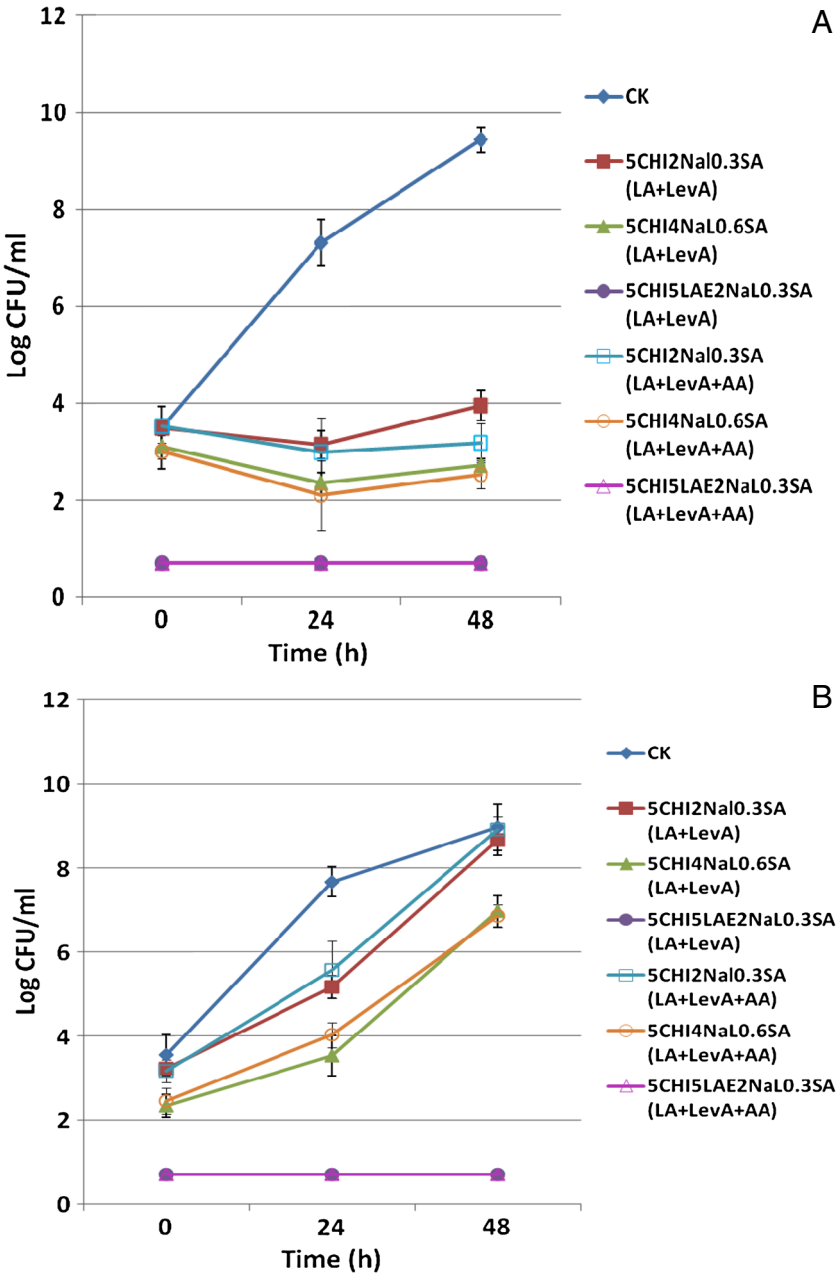

Fig. 4 Survivals of *L. innocua* (**a**) and *S.* Typhimurium (**b**) in TSB at 22 °C after film treatments containing chitosan, LAE, NaL, and SA. *LA* lactic acid, *LevA* levulinic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

The PLA film coated with 5CHI5LAE2NaL0.3SA (LA+LevA) and the PLA film coated with 5CHI5LAE (LA+LevA) were used to treat the surface-inoculated meat samples. The results are shown in Fig. 7.

At both inoculation levels, two films reduced around 1.5 log CFU/cm^2 of *Salmonella* and significantly inhibited the growth of *Salmonella* during storage of 3 or 5 weeks at 10 °C. At a higher initial population level, the survivals of *Salmonella* in the controls reached 5.5 log CFU/cm^2 after 7 days, while those in film-treated samples remained 3.8 log CFU/cm^2 from day 1 through day 21 (Fig. 7a), reducing approximately 1.7 log CFU/cm^2. Similar reduction was observed for meat samples with lower initial populations (Fig. 7b). There was no significant difference between the two films in the antimicrobial activity against *Salmonella* on meat, suggesting that the addition of NaL and SA at the tested concentrations did not make extra contribution to reduce or inhibit the growth of *Salmonella* on RTE meat, which is similar to *L. innocua* (Fig. 5) and *L. monocytogenes* (Fig. 6).

In all the experiments, the PLA film without antimicrobial coating (positive control) did not show any antimicrobial activity against both *Listeria* and *Salmonella* on meat samples (data not shown).

Discussion

The antimicrobial activities of organic acids and their salts are well documented (Doores 1993, 2002). Studies evaluating the

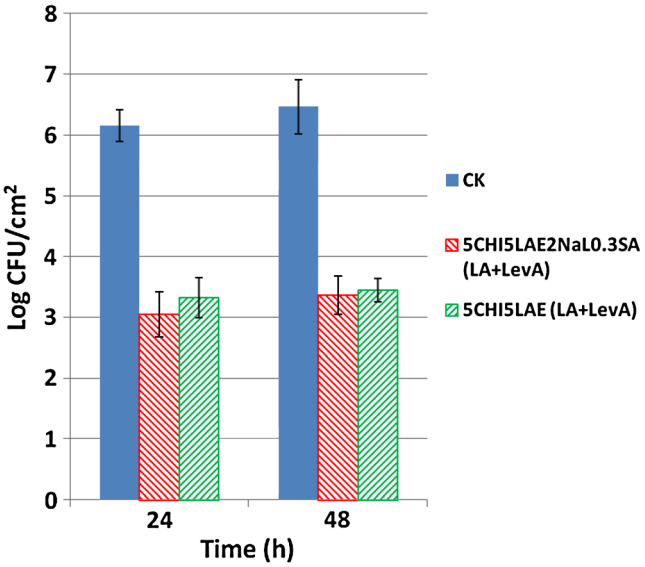

Fig. 5 Survivals of *L. innocua* on RTE meat after film treatments and storage at 10 °C for 2 days. *LA* lactic acid, *LevA* levulinic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

antimicrobial efficacy of acids in combination have been published (Grinstead and Angevaare 2003; Lemons 2009; Nou et al. 2011; Gurtler et al. 2012). However, there is limited information on their antimicrobial effect when used in combination for film coatings. In this study, the combinations of LA, LevA, AA, and CA were evaluated for their antimicrobial activity in a culture medium for screening trials. LA+LevA and LA+LevA+AA in coating solutions exhibited the highest antimicrobial activity among the test formulas. The addition of citric acid into coating solutions did not contribute more inhibitive effectiveness against *Listeria* (Fig. 1). These results agree with those reported by other researchers. Eswaranandam et al. (2004) reported that citric acid-incorporated films had less killing effect against *L. monocytogenes*. Zhuang et al. (1996) found that a cellulose-based edible film containing 0.2 and 0.4 % citric acid did not show any significant reduction of *Salmonella montevideo* on tomatoes.

The antimicrobial activity of chitosan is well document. However, when chitosan was used alone, its antimicrobial activity was limited, particularly when a low concentration was used (Fig. 1). Azevedo et al. (2014) reported that chitosan in a 1.5 % acetic acid solution did not show antimicrobial property. Perdonesa et al. (2012) observed that the use of pure chitosan film led to a slight reduction in the growth of *Botrytis cinerea* and this antifungal activity of chitosan films was enhanced by the addition of lemon essential oil. Duan et al. (2007) found that chitosan-alone coatings reduced 0.5 to 1.35 $\log_{10}$ CFU/g of *L. monocytogenes* and *E. coli* inoculated onto the surface of mozzarella cheese. Anacarso et al. (2011) also investigated the antilisterial activity of chitosan coatings on vegetables and fruits, and they found that chitosan alone generally impacted less than one log cycle of reduction. Therefore, a combination of chitosan with other antimicrobials is needed to achieve more bacterial reduction.

Sodium lactate is used in meat products as a flavor enhancer and also has been used as an antimicrobial to delay growth of meat spoilage organisms (Brewer et al. 1995; Maca et al. 1999; Vasavada et al. 2003) as well as food-borne pathogens (Miller and Acuff 1994; Mbandi and Shelef 2001; Bedie et al. 2001). Antimicrobial films with coating solutions containing 10 or 20 % NaL completely inhibited the growth of *Listeria* (Fig. 3a). However, the films were less effective in inhibiting *Salmonella* after 24 h (Fig. 3b). Houtsma et al. (1993) studied multiple pathogens and spoilage organisms occurring in meat products, and they concluded that Gram-positive bacteria were more sensitive toward lactate than Gram-negative bacteria. Gram-negative organisms are less susceptible to the action of antibacterials since they possess an outer membrane surrounding the cell wall that restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara 1992). A higher resistance of Gram-negative bacteria to other antimicrobial compounds has also been reported (Canillac and Mourey 2001; Delaquis et al. 2002; Harpaz et al. 2003). Torlak and Sert (2014) observed that the inhibitory efficiency of chitosan-coated polypropylene films against Gram-positive pathogens was higher than that against Gram-negative pathogens.

Increased antimicrobial effectiveness of organic acids may be achieved at lower concentrations with additional inhibitors, which is called hurdle concept (Leistner 1992). When the concentrations of NaL were reduced from 10 or 20 % to 2 or 4 %, respectively, while 0.3 or 0.6 % sorbic acid was added to the coating solutions, the antimicrobial films with coating solutions containing 4 % NaL and 0.6 % SA had equivalent antibacterial effectiveness (Fig. 4) to those containing 10 or 20 % NaL (Fig. 3), indicating the synergetic effect from the combination. Similar to the higher concentration of NaL used (Fig. 3), the combination of NaL+SA had more antimicrobial activity against *Listeria* than *Salmonella*.

LAE is a cationic surfactant and a derivative of lauric acid, L-arginine, and ethanol (Ruckman et al. 2004). LAE has a broad spectrum of antimicrobial activity (Bakal and Diaz 2005), and it has been classified as GRAS and a food preservative at a concentration of up to 200 ppm by the US Food and Drug Administration (Bakal and Diaz 2005). In this study, films with LAE at tested concentrations (1 and 2 %) reduced both *Listeria* and *Salmonella* in TSB from 3.5 log to undetectable without outgrowth during 48 h (Fig. 2). LAE in films exhibited more antimicrobial activity against both pathogens than NaL at tested concentrations (Figs. 3 and 4). Muriel-Galet et al. (2012) reported that EVOH copolymer films containing 5 and 10 % LAE produced total growth inhibition of *L. monocytogenes*, *E. coli*, and *S. enterica* in liquid culture media. Theinsathid et al. (2012) evaluated the antimicrobial

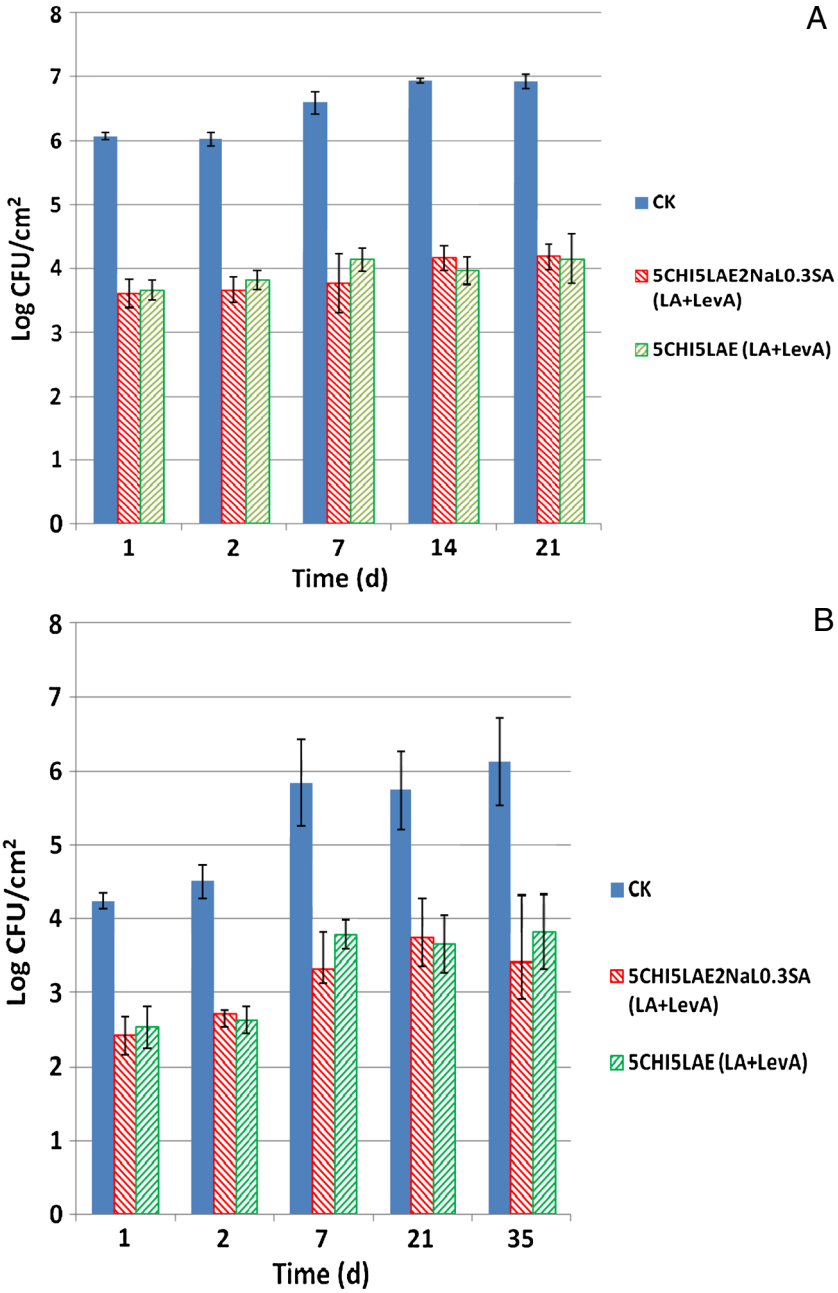

Fig. 6 Survivals of *L. monocytogenes* on RTE meat after film treatments and storage at 10 °C for 21 days (**a**) and 35 days (**b**). *LA* lactic acid, *LevA* levulinic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

activity of LAE-coated PLA films against *L. innocua* and *S.* Typhimurium inoculated on cooked ham and reported that those film treatments reduced both organisms by 2–3 log CFU/ml. Similar results were obtained in this study in TSB (Figs. 2 and 4) and on RTE turkey meat (Figs. 5, 6, and 7).

In this study, the same film treatments had less antimicrobial effectiveness against the microorganisms on the meat surface than that in TSB (Figs. 5 vs. 4a for *Listeria* and Figs. 7a vs. 4b for *Salmonella*). Numerous studies have reported that higher concentrations of antimicrobials or higher treatment doses are required in food systems to inhibit microorganisms than in growth media (Burt 2004; El-Shenawy et al. 1989; Kamat and Nair 1995; Karatzas et al. 2002; Patterson 1989; Shelef et al. 2006; Soni et al. 2010). Ma et al. (2013) observed that significantly higher concentrations of LAE are required to obtain similar inhibition of *L. monocytogenes* in milk than in TSB. Treating inoculated meat by combining nisin and gamma radiation, Mohamed et al. (2011) reported that these treatments achieved less reduction of *L. monocytogenes* in meat than in phosphate buffer. It could be explained that (1) the food matrix provides a protective environment to the pathogen as compared with phosphate buffer or other growth media; (2) food components interfere with antimicrobial activities by binding with antimicrobials; and (3) the meat surface has less moisture available as compared with liquid growth media, which reduces the release of antimicrobials from films to the meat surface.

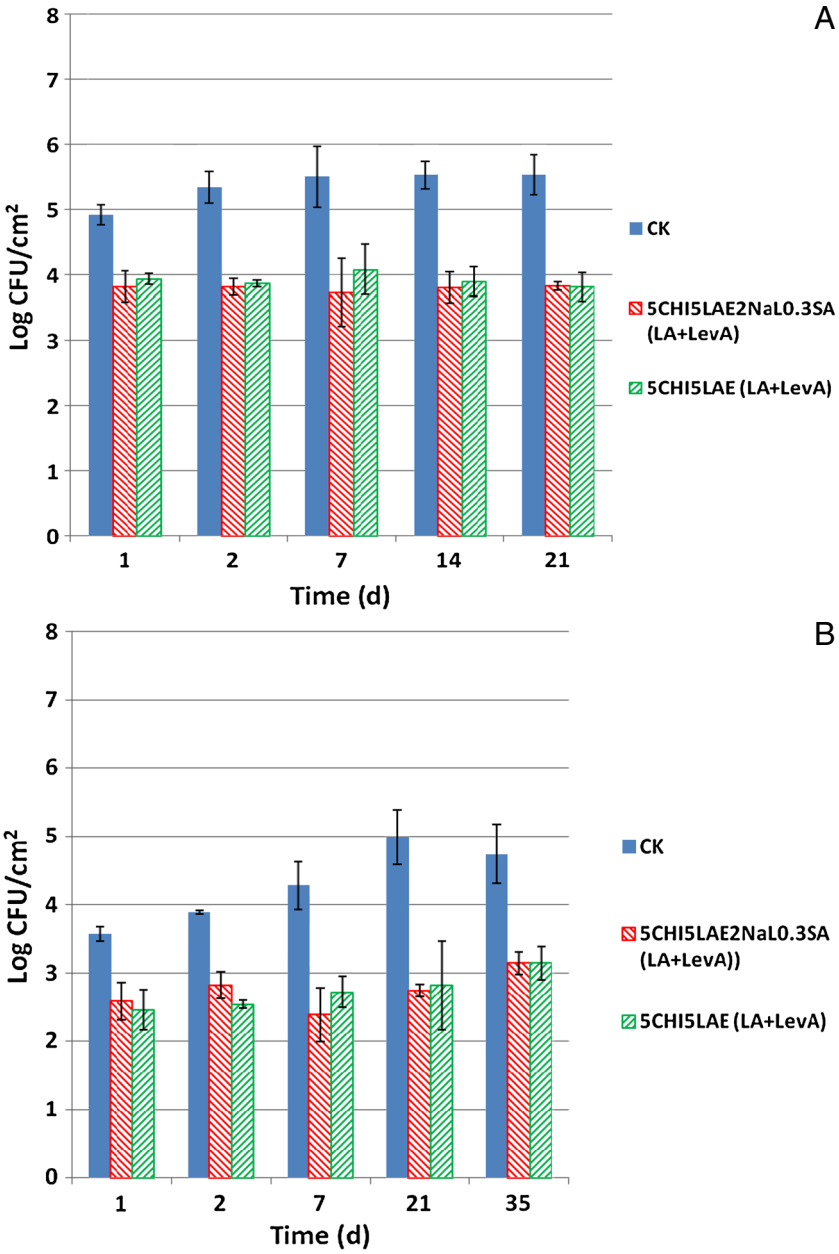

Fig. 7 Survivals of *S.* Typhimurium on RTE meat after film treatments and storage at 10 °C for 21 days (**a**) and 35 days (**b**). *LA* lactic acid, *LevA* levulinic acid. For other treatment codes, refer to Table 1. *Error bars* represent the standard deviation of the mean

In this study, we also observed that *L. monocytogenes* has similar sensitivity to antimicrobial film treatments as *L. innocua* in meat. Although *L. innocua* has been used as a model organism in various studies due to characteristics that are similar to those of the pathogenic species such as *L. monocytogenes* in terms of physiology and metabolism (Fairchild and Foegeding 1993; Francis and O'Beirne 1998; Gervilla et al. 1997; Ponce et al. 1998), the confirmation of the same level of antimicrobial effectiveness of PLA films on *L. innocua* and *L. monocytogenes* in this study would be valuable information for future challenge studies in pilot or commercial-scale facilities where pathogenic *L. monocytogenes* may be not allowed.

The contamination of RTE meat with *L. monocytogenes* and *Salmonella* spp. occurs mainly at post-processing, and consumption of these products without further heating is common. Although RTE meats contain salts such as sodium chloride, nitrite, and nitrate that have antimicrobial activities, they do not inhibit the growth of *L. monocytogenes* during storage at refrigeration temperatures (Mbandi and Shelef 2002). Therefore, additional intervention technologies are needed to enhance the safety of RTE foods. The present study demonstrates the antimicrobial efficacy of PLA films in combination with chitosan and acids against *Listeria* and *Salmonella* in culture medium as well on RTE meat. To our knowledge, this is the first study published evaluating antimicrobial effectiveness using a PLA film in combination with chitosan, multiple acids, and other antimicrobials. Two application methods of antimicrobial packaging have been studied to inactivate pathogens

Table 2 Application methods of antimicrobial packaging

Polymer	AM	Incorporation of AM	Target microorganism	Test medium	Application method	Reference
Chitosan	AIT	Coating solution	*Salmonella*	Cantaloupe	Direct coating	Chen et al. (2012)
Gelatin	Lysozyme, nisin, EDTA	Coating solution	*E. coli*, *Listeria*, *Salmonella*	RTE meat	Direct coating	Gill and Holly (2000)
Chitosan	Acids	Coating solution	*Salmonella*	Tomato	Direct coating	Jin and Gurtler (2012)
Alginate, pectin, κ-carrageenan	Nisin, NaL, NaD, PS	Coating solution	*Listeria*	RTE meat	Direct coating	Juck et al. (2010)
Zein	Nisin, NaL, NaD	Coating solution	*Listeria*	RTE meat	Direct coating	Lungu et al. (2005)
Gelatin	Nisin	Coating solution	*Listeria*	CM, bologna	Direct coating	Min et al. (2010)
Polyvinyl-alcohol	Lysozyme	Composite film	*A. acidoterrestris*	CM, juice	Film packaging	Conte et al. (2006)
Pectin	Nisin	Composite film	*Listeria*	RTE meat	Film packaging	Jin et al. (2009a)
Pectin, PLA	Nisin	Composite film	*Listeria*	CM, juice, liquid egg	Film packaging	Jin et al. (2009b)
PLA	Nisin	Composite film	*Listeria*, *E. coli*, *Salmonella*	CM, juice, liquid egg	Film packaging	Jin and Zhang (2008)
PLA	Nisin, EDTA	Composite film	*E. coli*	CM	Film packaging	Liu et al. (2010)
PLA	Nisin	Composite film	*Listeria*	CM	Film packaging	Liu et al. (2009)
EVOH copolymers	LAE	Composite film	*Listeria*, *E coli*, *Salmonella*	CM, milk	Film packaging	Muriel-Galet et al. (2012)
Chitosan, PLA	LAE, NaL, SA	Coating on PLA film surface	*Listeria*, *Salmonella*	CM, RTE meat	Film packaging	This study

AM antimicrobials, *AIT* allyl isothiocyanate, *NaL* sodium lactate, *NaD* sodium diacetate, *PS* potassium sorbate, *LAE* lauric arginate, *SA* sorbic acid, *EDTA* ethylenediaminetetraacetic acid disodium salt, *PLA* polylactic acid, *CM* culture media

and spoilage microorganisms in foods and culture media: direct coating and film packaging, as summarized in Table 2. Direct coating methods (an edible coating is applied by dipping, brushing, or spraying onto the food) have been reported (Cagri et al. 2004; Chen et al. 2012; Gill and Holley 2000; Jin and Gurtler 2012; Juck et al. 2010; Lungu and Johnson 2005; Min et al. 2010). The advantage of the antimicrobial PLA film developed in this study over the direct coating method is that antimicrobial coating films can be customized within current film manufacturing lines, premade by a packaging company, and used as a regular packaging material, while preparation of coating solutions and coating/drying procedures for direct coating on-site might slow down production speed. As shown in Table 2, this antimicrobial coating PLA film has the same application method as one-step composite antimicrobial PLA films (incorporate antimicrobials directly into polymer materials to make composite antimicrobial films) (Conte et al. 2006; Jin et al. 2009a, b; Jin and Zhang 2008; Liu et al. 2010, 2009; Muriel-Galet et al. 2012); however, there are also advantages of this antimicrobial-coated PLA film over the composite films. These advantages include (1) less concern of the loss of antimicrobial activity during thermal film making, (2) reduced amount of antimicrobial compounds used in packaging material since only the outer layer plays an antimicrobial part; and (3) avoidance of "filler effect" and minimal impact on physical or mechanical properties of base materials (Li et al. 2012). This study provides RTE food processors different approaches for the control of *Listeria* and *Salmonella* in packaged RTE foods. Nevertheless, there are still some challenges in the application of the developed antimicrobial coating films in the meat industry: the antimicrobial activity of the active coating layer may be affected by storage and handling conditions and the loss of physical stability of the films, such as separation of the active coating layer from the base PLA film,

may be a concern during storage and transportation, which needs to be further investigated.

Conclusions

The antimicrobial activities of chitosan-coated PLA films containing multiple organic acids and other antimicrobials were clearly demonstrated against *L. innocua*, *L. monocytogenes*, and *S.* Typhimurium with significant inhibition of microbial growth in the culture medium during 48 h at 22 °C. Gram-positive *Listeria* was more sensitive to these film treatments than Gram-negative *Salmonella*. The antimicrobial PLA films significantly inhibited the growth of both microorganisms on RTE meat during storage at 10 °C for up to 5 weeks and indicated a potential application of this antimicrobial packaging system in meat products. Further research on higher concentrations of antimicrobials in film coatings used for meat samples is needed to achieve higher reduction of the pathogens. Alternatively, using nanotechnology to encapsulate chitosan and antimicrobials could be an effective approach to enhance the antimicrobial efficiency of the antimicrobial packaging films. Pretreatment of the PLA film surface before coating to improve the physical stability of the films should also be considered.

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